

Addendum

The cuticle: Not only a barrier for plant defence

A novel defence syndrome in plants with cuticular defects

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The cuticle is a physical barrier that prevents water loss and protects against irradiation, xenobiotics and pathogens. This classic textbook statement has recently been revisited and several observations were made showing that this dogma falls short of being universally true. Both transgenic *Arabidopsis thaliana* lines expressing cell wall-targeted fungal cutinase (so-called CUTE plants) or lipase as well as several *A. thaliana* mutants with altered cuticular structure remained free of symptoms after an inoculation with *Botrytis cinerea*. The alterations in cuticular structure lead to the release of fungitoxic substances and changes in gene expression that form a multifactorial defence response. Several models to explain this syndrome are discussed.

Expression of a Fungal Cutinase Leads to a Strong Immune Response to *B. cinerea*

Heterologous overexpression of a cell wall-targeted fungal cutinase of *Fusarium oxysporum* in *A. thaliana* provides total immunity to *B. cinerea*.¹ The protective effect in these CUTE plants was dependent on the enzymatic activity of the protein, since plants transformed with genes mutated in the active site of the cutinase are no longer protected.¹ Plants transformed with lipase A of *B. cinerea* also exhibit full protection, confirming the importance of the cutinolytic activity for the syndrome observed in CUTE plants.¹ Protection is also taking place in plants treated ectopically with cutinase of *F. solani*. A direct toxic effect against *B. cinerea* of cutinase or products of the action of cutinase on the cuticle could be excluded¹ and the possible involvement of induced plant defences in this response was tested as a first hypothesis. The expression of marker genes *PR-1*, *PR-3*, *PR-4* and *PDF1.2* for the salicylic acid (SA), ethylene (E) or jasmonic acid (JA) pathways were determined. No correlation was observed that might link induced resistance with resistance in CUTE plants.¹ The cutinase gene of *F. solani* was also expressed in *A. thaliana* mutants of the SA (*pad4*, *sid2*), ET (*etr1*, *ein2*, *pad2*) and

of the JA (*jar1*) pathways, but the resistance to *B. cinerea* observed in CUTE plants is fully independent of SA, ET and JA.¹ The idea was further tested if other genes might be linked to resistance in CUTE plants using genome-wide microarrays. Changes in gene expression in *B. cinerea*-infected CUTE plants were compared to *B. cinerea*-infected control plants and 15 genes were selected on the basis of an earlier and stronger expression after inoculation with *B. cinerea*. The expression and accumulation of the products of such genes might explain, at least partially, the full immunity observed in CUTE plants. Constitutive overexpression of each of these genes in separate plants showed that 8 out of 15 genes had a statistically significant effect on the tolerance of the plants to *B. cinerea*. The genes included three members of the lipid transfer protein (LTP), two members of the peroxidase (PO) and two members of the protein inhibitor (PI) gene families respectively.¹ Members of the LTP gene family have previously been shown to be toxic in vitro against various fungi² and overexpression of LTP from pepper provides resistance to *B. cinerea* when expressed in *A. thaliana*.³ POs were proposed to reinforce cell walls most likely by crosslinking lignin monomers^{4,5} and one putative role for proteinase inhibitors might be to interfere with hydrolytic fungal enzymes involved in the penetration process.⁶ Tobacco overexpressing a PI gene from *Nicotiana glauca* is better protected against *B. cinerea*.⁷ The microarray analysis also revealed changes in gene expression of other genes. For example, changes in the expression of genes encoding for the polygalacturonase-inhibiting proteins *AtPGIP1* and *AtPGIP2* were also observed. *AtPGIP2* is expressed constitutively in CUTE plants, and is induced 36 h after inoculation of WT plants with *B. cinerea*. Accordingly, extracts of CUTE plants contained a strong PGIP activity in comparison to extracts from WT plants, demonstrating that CUTE plants produce PGIPs prior to infection.⁸ This is interesting, since PGIPs have been linked to the generation of oligogalacturonide monomers by partially inhibiting the action of fungal polygalacturonase released during the infection process.⁹ For instance, overexpression of both *AtPGIP1* and *AtPGIP2* genes in *Arabidopsis* led to an increase in tolerance to *B. cinerea*.¹⁰ However, overexpression of the CUTE gene in transgenic plants expressing an antisense PGIP did however not lead to increased resistance to *B. cinerea*.⁸ Thus, members of the LTPs, PER and PIs might contribute to the resistance induced by *B. cinerea* in the cuticle altered CUTE plants, while PGIPs might contribute but are not essential for the defence in CUTE plants.

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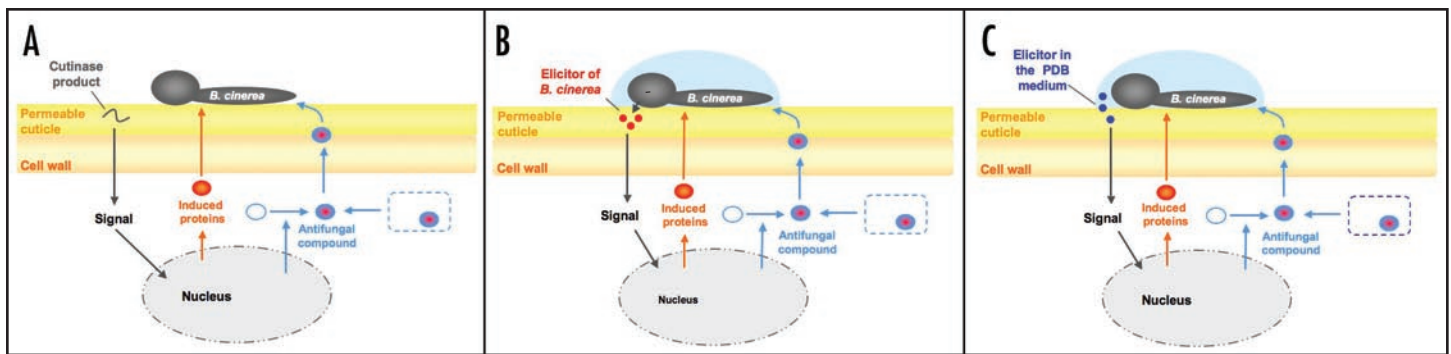


Figure 1. Schematic representations of three scenarios explaining an increased tolerance to *B. cinerea*. The first model (A) is based on the release of cutin monomers that function as a signal for induced resistance in the plant cell. The response of the plant includes the production of antifungal and other protective proteins as well as the accumulation of an antifungal compound (either by de novo synthesis or release from a compartment). The two other models capitalize on the increased permeability of the cuticle and propose that either elicitors *B. cinerea* (B) or from the PDB (potato dextrose broth) incubation medium (C) diffuse readily in the cell where they activate the same defences as in (A).

A second hypothesis might be that CUTE plants accumulate and release a fungitoxic metabolite that interferes with fungal growth. The presence of a fungitoxic activity was indeed observed in the inoculation droplets of the *B. cinerea* spore suspension.¹ Gel filtration chromatography and other analyses indicate that the putative substance has a molecular weight smaller than 5000 D, is charged and heat resistant.⁸ Digestion with protease K reduced the fungitoxic activity of the diffusate.⁸ At the present the chemical nature of the leaf diffusate remains to be determined.

Mutants Altered in Cuticular Structure Exhibit a Similar Syndrome as CUTE Plants

The intriguing observation made with CUTE plants was completed by a study on a series of mutants impaired in various aspects of cuticle formation. The *lcr* (*lacerata*) mutant has a defect in a gene coding for a cytochrome P450 monooxygenase involved in the formation of ω -hydroxy fatty acids in yeast and could be involved in cutin biosynthesis.¹¹ The *lacs2* (*long-chain acyl-CoA synthetase*) also identified as *bre1*¹² (*Botrytis resistant*) mutant has a thinner cuticle than WT plants with a strong reduction in dicarboxylic acid monomers in the cutin polyester.^{12,13} The *acel/hth* (*adhesion of calyx edges / hothead*) mutant, deficient in fatty acid ω -alcohol dehydrogenase activity, shows a reduction in the levels of the major constituents of cuticular polyesters and cutin.¹⁴ The *bdg* (*bodyguard*) mutant accumulates more cell wall-bound lipids and epicuticular waxes than WT plants.¹⁵ Interestingly an increase in resistance to *B. cinerea* was observed in the *lcr*, *lacs2/bre1* and *bdg* mutants but not in the *acel/hth* mutant. In the case of *lcr* and *bdg* the resistance correlates with the expression of the same genes as identified in the CUTE plants.⁸ However, in the case of other mutants showing an increased resistance to *Botrytis* (such as *lacs2/bre1*) this correlation was not observed, indicating that other factors must be involved in the resistance to *Botrytis*.⁸ A fungitoxic activity could also be observed in the *lcr*, *lacs2/bre1* and *bdg* mutants.^{8,12} No fungitoxic diffusate was observed in the *acel/hth* mutant. The accumulation of a fungitoxic activity could be correlated with the permeability of the cuticle. For instance, the cuticular permeability of CUTE, *lcr*, *lacs2/bre1*, *bdg*^{8,12} is higher than that of WT controls, whereas no difference was observed between *acel/hth* mutants and WT plants.¹² Interestingly, Tang et al¹⁶ also recently observed an association between resistance to *Botrytis* and increased permeability of mutants

in the *LACS* gene. Thus, the presence of a fungitoxic activity appears to be mostly associated with an increase in cuticular permeability.

The Cuticle: Source of Signals for Defence or Barrier for Diffusion

Three scenarios might explain the resistance of plants with defective cuticles (Figs. 1A–C). In the first model (Fig. 1A), the putative products of the cutinase, will act as intracellular signals and trigger multifactorial defences. Potentially, some of such monomers might also be produced in cuticular mutants as a result of incomplete cuticle formation. These defences might involve the production of antimicrobial proteins and the production or release of antifungal metabolites. In the second model (Fig. 1B), the permeable cuticle of CUTE plants or of cuticle mutants would allow a better uptake of putative elicitors of *B. cinerea* into the leaf cells. The elicitors might trigger a faster and more intensive defence reaction. Finally, in the third model (Fig. 1C) the elicitors for defence reactions would originate from the PDB (potato dextrose broth) medium used for the inoculation of *B. cinerea*. The surprising potential for defence against *B. cinerea* unveiled in CUTE plants warrants further research to understand its molecular basis. Remarkably, *A. thaliana* is exposed to cutinase and lipase A produced by *B. cinerea* during infection, yet no resistance is visible. Perhaps the timing or the quantity of enzyme produced is not appropriate for resistance to be induced. Alternatively, *B. cinerea*, like other pathogens, might suppress induced defence response in the plant.

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